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# A Protein Tyrosine Phosphatase Expressed within Dopaminoceptive Neurons of the Basal Ganglia and Related Structures

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**Immunocytochemical and biochemical studies were conducted to characterize a brain-specific protein tyrosine phosphatase, designated STEP for striatal enriched phosphatase. STEP immunoreactivity was most intense in select regions of the CNS receiving a dopaminergic input, and was localized to cell bodies, dendrites, and axonal processes. Western blot analyses of rat brain homogenates revealed a triplet of polypeptides with relative mobilities ($M_r$) of 46 kDa, 37 kDa, and 33 kDa enriched within the striatum. Phase separation of protein homogenates by Triton X-114 extraction indicated that this triplet was enriched in soluble but not membrane fractions. Affinity-purified STEP fusion protein exhibited phosphatase activity while a mutated form of the STEP fusion protein (Cys300Ser) showed no demonstrable phosphatase activity.**

***[Key words: protein tyrosine phosphatase, dopamine, striatum, caudatoputamen, basal ganglia, DARPP-32, tyrosine hydroxylase, intracellular phosphatase]***

Protein phosphorylation is one of the chief mechanisms by which molecular signals within neurons can be regulated (Greengard, 1987; Hemmings et al., 1989; Huganir and Greengard, 1990; Walaas and Greengard, 1991). A majority of brain phosphoproteins are phosphorylated by serine and threonine kinases and dephosphorylated by serine and threonine phosphatases. Some of these brain phosphoproteins are localized within functionally distinct cell types, as exemplified by the group of dopamine- and/or cAMP-regulated phosphoproteins, and several members of this family are expressed selectively by dopaminoceptive neurons of the basal ganglia (Ouimet et al., 1984, 1989; Walaas et al., 1984a,b; Nairn et al., 1988; Girault et al., 1990).

In contrast, phosphoproteins that are phosphorylated on tyrosine residues constitute a minority of brain phosphoproteins. It is unclear whether any of these phosphoproteins are restricted to functionally distinct neural circuits or neurotransmitter pathways; however, this has been suggested by several lines of evidence. First, some proteins within striatal neurons are specifically phosphorylated on tyrosine residues after loss of dopamine inputs from the substantia nigra, suggesting that tyrosine phosphorylation may underlie some of the postsynaptic effects of dopamine (Girault et al., 1992a). Second, a number of novel protein tyrosine phosphatases (PTPs) have been identified on pioneer axons pathways in the *Drosophila* embryo (Tian et al., 1991; Yang et al., 1991), suggesting a role of PTPs in axon navigation during development.

Two families of PTPs have been identified on the basis of their structural organization (Hunter, 1989; Alexander, 1990; Fischer et al., 1991). Members of the first family are receptor-like PTPs with a large extracellular domain, a single transmembrane domain, and an intracellular region composed of two phosphatase domains. This motif is found among nearly all receptor-like PTPs (Streuli et al., 1988; Jirik et al., 1990; Kaplan et al., 1990; Matthews et al., 1990; Sap et al., 1990). Members of the second family are intracellular, nonreceptor PTPs with a single phosphatase domain. PTP1B was the first PTP of this family to be isolated, purified, and characterized (Tonks et al., 1988a,b; Charbonneau et al., 1989). Additional members of the intracellular family have now been isolated and characterized (Cool et al., 1989; Guan et al., 1990; Gu et al., 1991; Shen et al., 1991; Yang and Tonks, 1991).

A new member of the intracellular family of PTPs, designated STEP (striatal enriched phosphatase), was shown to be brain enriched (Lombroso et al., 1991). The STEP 3 kilobase (kb) cDNA clone encodes a 369 amino acid polypeptide with a predicted molecular weight of 42 kDa, and contains a 12 amino acid sequence identical to the canonical sequence found within the phosphatase domain of all PTPs isolated to date (Charbonneau et al., 1989; Krueger et al., 1990).

Northern analyses reveal that STEP mRNA is expressed exclusively within the CNS. Furthermore, STEP mRNA shows a distinctive distribution in the brain, with the highest levels of expression in the striatum, but is also present to a lesser extent in the cerebral cortex, and is not detectable in the cerebellum (Lombroso et al., 1991). The present study was carried out to determine whether STEP polypeptides are associated with distinct neuronal classes or neurotransmitter systems within the basal ganglia and to assess the phosphatase activity and the biochemical properties of the STEP protein.

## Materials and Methods

*Construction and purification of fusion protein.* The bacterial expression vector pGEX-2T (Smith and Johnson, 1988) was used to produce STEP protein fused to glutathione *S*-transferase (GST). As there are three methionines within 40 amino acids of the N-terminal, it was unclear

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which served as the actual initiation site. Therefore, a fusion protein was constructed starting at position amino acid 41, immediately after the third methionine. The nucleotide sequence encoding for the carboxyl 329 amino acids of the STEP protein was subcloned in-frame at the SmaI restriction site of the pGEX-2T polylinker region and recombinants were transformed into competent cells. Standard protocols were followed for the purification of fusion protein, mutant fusion protein (see below), or control GST protein (Smith and Johnson, 1988). In brief, bacteria with either the recombinant or parental vector were grown at 37°C in 500 ml of Luria-Bertani (LB) broth containing 50 μg/ml ampicillin to an optical density ($OD_{550}$) of 0.8, and induced by the addition of 0.5 ml of 100 mM isopropyl-β-galactopyranoside (IPTG) for 5 hr. The bacteria were pelleted, resuspended in 10 ml of ice-cold 50 mM imidazole, pH 7.2, 0.1% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and lysed using a sonicator with a 2 mm diameter for 30 sec. Insoluble material was spun down and the supernatant was affinity purified by a batch procedure using 2 ml of glutathione-agarose beads (50% slurry; Sigma), and eluted using 50 mM Tris, 5 mM reduced glutathione. Fractions were pooled, dialyzed against imidazole buffer, and analyzed by SDS-PAGE. The expected size of the recombinant protein was approximately 65 kDa (see inset, Fig. 2). The average yield was 1 mg fusion protein per liter of bacteria. For enzymatic assays, aliquots were brought to 50% glycerol and frozen at −80°C.

*Construction of mutant STEP (Cys300Ser) fusion protein.* A single nucleotide missense mutation was introduced into the STEP cDNA clone by polymerase chain reaction (PCR) mutagenesis in order to convert the cysteine at amino acid position 300 to a serine. Two 24 nucleotide oligomers were synthesized across the phosphatase domain of interest in both sense and antisense orientation (PL10.sense = TCATTGTTCACTCCAGTGCAGGGA). The sequence in boldface is the codon for serine rather than the original cysteine (TGC) at STEP nucleotide positions 1634–1636 (Lombroso et al., 1991). These oligomers were then paired with either a 5′, 30 base pair (bp) oligomer (PL11.sense = TGACTGTCAAGTCCATGGGTCTGCAGGAGA) located at STEP nucleotides 843–872, or a 3′ 26 bp oligomer (PL2.anti = TAACACATGTTGCCAAAGAGTCATGC) located at STEP nucleotides 2664–2689. PCR was performed following manufacturers protocol using the original STEP cDNA clone as template. The reaction produced the expected 800 bp 5′ fragment and 1000 bp 3′ fragment, each of which contained an overlapping 24 bp region (PL10). Approximately 10 ng of each purified band was mixed together, denatured by heating to 94°C, and reannealed at 60°C. Tac polymerase enzyme was used to extend the overlapping sequence in either direction. A PCR reaction was repeated with the 5′ oligomer (PL11) and the 3′ oligomer (PL2) for 30 cycles at 94°C, 60°C, and 72°C for 1, 2, and 3 min, respectively. The 1.8 kb fragment produced was digested at unique SacI and SalI sites to produce a 1 kb fragment. This fragment was ligated into pGEX-STEP, replacing the equivalent 1 kb SacI/SalI fragment and transformed into competent TG1 bacteria. Sequencing through the entire open reading frame confirmed that only a single nucleotide had been changed, converting Cys at amino acid position 300 to Ser. The mutated fusion protein was then affinity purified as described above.

*Phosphatase assays. para*-Nitrophenyl phosphate (pNPP) was used as the substrate in enzymatic assays for tyrosine phosphatase activity. Assays were carried out in the presence of 50 mM imidazole buffer, pH 7.0, with 2.5 mM pNPP for 30 min at 30°C, and the reaction was terminated by the addition of 900 μl of 0.2 *N* NaOH. Results are expressed as the concentration of *p*-nitrophenolate ion produced in the reaction using a molar extinction coefficient of $1.78 \times 10^4\ M^{-1}\ cm^{-1}$ at 410 nm.

*Polyclonal antibody production and affinity purification of antisera.* STEP polyclonal antibodies were raised in two rabbits immunized with an 18 amino acid peptide synthesized on a 430A Applied Biosystems Peptide Synthesizer, and purified by HPLC. The amino acids comprising the peptide were selected from an N-terminal region showing no homology to other known tyrosine phosphatases identified to date (amino acids 57–74; Lombroso et al., 1991). Two female New Zealand White rabbits were each injected intradermally at multiple sites with a total of 300 μg of purified peptide (0.75 ml), conjugated to rabbit serum by glutaraldehyde. For the initial immunization, the antigen was emulsified with an equal volume (0.75 ml) of Freund's complete adjuvant. The rabbits were boosted subcutaneously 4 weeks later, and at 2 week intervals thereafter for 4 months, with 40 μg peptide in PBS, pH 7.4. Prior to each injection, 10 ml of serum was obtained by venipuncture, and immunologic response was monitored by ELISA assays. The rabbits were then deeply anesthetized with pentobarbital before being exsanguinated, and all serums were collected and stored at −20°C in 0.1% Na azide. Affinity purification was performed by first passing the serum over a 10 ml protein A column, followed by extensive dialysis of the eluted IgG fractions against 0.05 M Na bicarbonate, pH 7.5, and subsequent immunoabsorption on a 2 ml column containing 1 mg of purified STEP fusion protein bound to cyanogen bromide–activated sepharose beads (Pharmacia). Purified STEP antiserum was eluted using 3 M guanidinium and dialyzed against 0.05 M sodium bicarbonate. Fractions showing highest immunoreactivity for STEP fusion protein by ELISA assays were pooled and stored at −80°C.

*Western blotting.* Ten and twelve percent polyacrylamide SDS gels were used for analysis of STEP immunoreactive proteins according to the method of Laemmli (1970). Adult Long Evans rats (male and female) were decapitated, their brains removed, and brain regions rapidly dissected and frozen in liquid nitrogen until further needed. Protein homogenates were made by sonication for 1 min in sonication buffer [10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM PMSF (Sigma), 0.1% soybean trypsin inhibitor (Sigma), 0.1% aprotinin (Sigma), 1 mM EDTA, 1 mM EGTA]. In parallel experiments, the effects of 150 mM NaCl or 5 mM CaCl (with no EDTA/EGTA) were investigated, and gave essentially results identical to those shown in Figure 2. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumen as the standard. Samples were briefly spun to remove insoluble material, aliquoted, and frozen in liquid nitrogen until further use. Fifty to one hundred micrograms of protein of whole rat brain, striatum, cerebral cortex, or cerebellum were loaded per lane, separated by electrophoresis, and transferred to nitrocellulose filters for Western blotting.

Protein extractions with Triton X-114 were conducted essentially as previously described (Bordier, 1981), with minor modifications. In brief, protein homogenates were adjusted to a concentration of 1 mg/ml with PBS, and 1 ml used for each experiment. Triton X-114 was added to a final concentration of 1% and stirred on ice for 15 min. After spinning to remove undissolved particles, the supernatant was heated to 30°C for 5 min, placed over a 1 ml sucrose cushion (6% sucrose in PBS, 0.06% Triton X-114), and spun at 3000 rpm. The detergent phase is found as an oily droplet at the bottom of the tube and is separated from the aqueous phase. The supernatant was extracted once more with Triton X-114. Proteins were precipitated using 100% ice-cold acetone, and the pellet washed with 50% acetone and resuspended in sample buffer, boiled, and loaded onto SDS-acrylamide gels.

For detection of immunoreactive bands, blots were processed as described (Naegele and Barnstable, 1991). Briefly, blots were incubated in blocking buffer for 30–60 min and then incubated overnight in affinity-purified polyclonal antisera diluted 1:50. Controls included incubation with antisera preincubated with STEP fusion protein. The blots were then washed, incubated in alkaline phosphatase–conjugated anti-rabbit IgG (Promega, 1:5000), and washed again, and bands were visualized by incubating blots in nitro-blue tetrazolium (NBT; Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma). To detect synaptophysin immunoreactivity, membranes were incubated with a monoclonal antibody (mAb; gift of R. Jahn) diluted 1:1000 and processed as above, substituting anti-mouse IgG (Promega, 1:5000) for the secondary antibody.

*Immunoperoxidase immunocytochemistry.* Adult Long Evans rats (male and female) were anesthetized with pentobarbital (Nembutal; 40 mg/kg, i.p.) and perfused through the left ventricle with approximately 50 ml of PBS followed by 200–300 ml of PBS containing 4% paraformaldehyde at room temperature. Brains were removed after 1 hr and blocks of tissue were cryoprotected in 10%, 20%, and 30% sucrose in phosphate buffer overnight. The blocks were then frozen in OCT compound and stored at −80°C until use. For free-floating staining, 50-μm-thick vibratome sections were cut in coronal and horizontal planes from tissue blocks after thawing in ice-cold PBS. Reduction of nonspecific staining and enhancement of antibody penetration was accomplished by incubating sections in 5% normal serum in PBS containing 0.4% Triton X-100 for 1 hr at room temperature. Sections were then washed and incubated on a shaker for 24 hr at 4°C in affinity-purified STEP antiserum, diluted 1:75–1:100, or in mouse monoclonal antibody against DARPP-32 (gift of G. Snyder and P. Greengard), diluted 1:400,000–1:500,000, or in mouse monoclonal antibody against tyrosine hydroxylase (TH; Boehringer Mannheim) diluted 1:200. Specificity for STEP antiserum was checked by blocking experiments, consisting of preincubating the antibody diluted 1:75 for 2 hr at 4°C with 16 μg STEP fusion protein or 4 μg/ml of STEP peptide. Free-floating vibratome sections were then

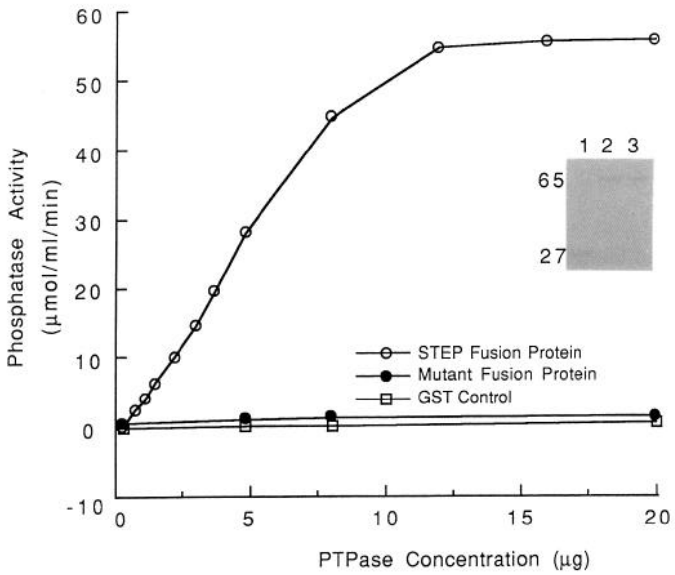


*Figure 1.* Comparison of normal and mutant STEP fusion protein in a phosphatase assay. STEP fusion protein was affinity purified and assayed for phosphatase activity against the substrate pNPP (○). The activity of a mutant STEP fusion protein, in which a cysteine at amino acid position 300 was converted to serine, is also shown (●). This cysteine, found in all PTPs isolated to date, has been suggested as critical for enzymatic activity. The enzymatic activity of both were compared to the control fusion protein, GST alone (□). The *inset* shows the relative mobilities of approximately 1 μg of each purified construct after electrophoresis on a 12% SDS-acrylamide gel and Coomasie staining. *lane 1,* GST control protein; *lane 2,* mutant STEP fusion protein; *lane 3,* STEP fusion protein.

immunostained using this mixture rather than STEP antisera. Sections were processed for immunoreactivity using biotinylated anti-mouse or anti-rabbit IgG and an avidin-biotin-peroxidase complex (Vector) (Naegele and Barnstable, 1991).

*Two-color immunofluorescence staining for STEP and DARPP-32.* Six adult Sprague–Dawley rats were used for double-labeling studies. Animals were deeply anesthetized and perfused as described above. The brains were removed, blocked in the coronal plane, and cryoprotected for 1 hr in 10% sucrose and overnight in 20% sucrose. The following day, the blocks were equilibrated in 30% sucrose, embedded in OCT compound and frozen on dry ice. Ten-micrometer-thick cryostat sections of rat caudatoputamen and neocortex were cut in the horizontal plane. The cryostat sections were stored at −80°C for up to 4 weeks before use. Prior to double labeling, slides were warmed to room temperature, and nonspecific staining was blocked by incubation in blocking buffer (PBS containing 3% goat and 3% horse serum; Vector Labs) for 1 hr. Sections were then incubated overnight at room temperature in a cocktail containing rabbit anti-STEP (1:25–1:50) and either mouse monoclonal anti-DARPP-32 (1:100,000) or mouse anti–tyrosine hydroxylase (1:100) diluted in the blocking buffer. These dilutions were found to give optimal double labeling with low background in initial pilot experiments. Since sequential incubations in each antibody did not produce staining patterns markedly different from simultaneous incubations in a cocktail of the two antibodies, the latter method was used. Subsequently, sections were washed three times in blocking buffer and incubated for 1 hr at room temperature in secondary antibodies. STEP immunoreactivity was detected using biotinylated secondary antibodies directed against rabbit immunoglobulins (goat anti-rabbit biotin; Vector Labs) and avidin-neutralite fluorescein (Molecular Probes). DARPP-32 or tyrosine hydroxylase immunoreactivity was localized with a horse anti-mouse IgG conjugated to rhodamine (Boehringer Mannheim). After final rinsing in PBS, sections were coverslipped in PBS:glycerol (1:1) and viewed under epifluorescence illumination in a Zeiss Axiovert microscope equipped with a dual filter for simultaneous rhodamine and fluorescein visualization (Omega). Comparisons and counts of single- and double-labeled neurons were carried out at magnifications of 400–600×.

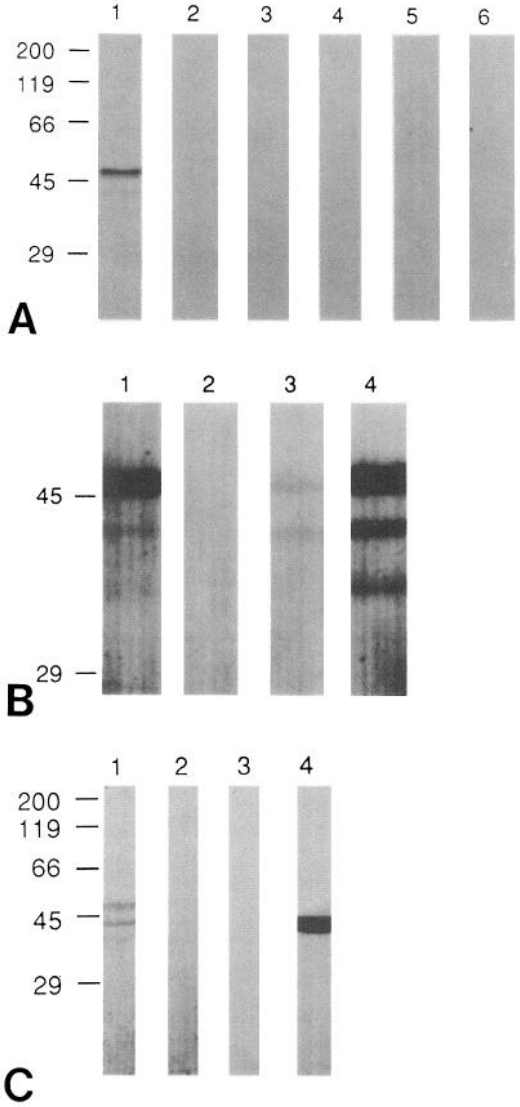


*Figure 2.* Western blots showing STEP immunoreactivity enrichment in certain brain regions. Total brain homogenates are compared to various peripheral tissues in *A*: total brain (*lane 1*), heart (*lane 2*), kidney (*lane 3*), liver (*lane 4*), and spleen (*lane 5*). STEP immunoreactivity in brain is completely blocked by preincubation of antisera with STEP fusion protein (*lane 6*). In *B,* different brain regions are compared: total brain (*lane 1*), cerebellum (*lane 2*), cerebral cortex (*lane 3*), and striatum (*lane 4*). Three immunoreactive bands of molecular weights 46 kDa, 37 kDa, and 33 kDa are enriched in striatum. In *C,* phase separation by Triton X-114 demonstrated that STEP immunoreactivity is associated with soluble fractions but not membrane fractions: aqueous phase (*lanes 1* and *3*), Triton phase (*lanes 2* and *4*). Membranes were immunoblotted with either STEP antisera (*lanes 1* and *2*) or mAb against an integral membrane protein, synaptophysin (*lanes 3* and *4*), as a control. Approximately 100 μg of protein homogenates per lane was electrophoresed on 12% SDS-acrylamide gel transferred to nitrocellulose membranes, and immunoblotted as described in the Materials and Methods. Blots were scanned and digitized to enhance contrast of fainter bands.

## Results

### *STEP has tyrosine phosphatase activity*

In order to demonstrate tyrosine phosphatase activity, a fusion protein was constructed consisting of glutathione *S*-transferase linked to STEP (GST-STEP) and affinity purified (Fig. 1, inset). The fusion protein was shown to have phosphatase activity, as is shown in Figure 1, with pNPP used as substrate. As pNPP

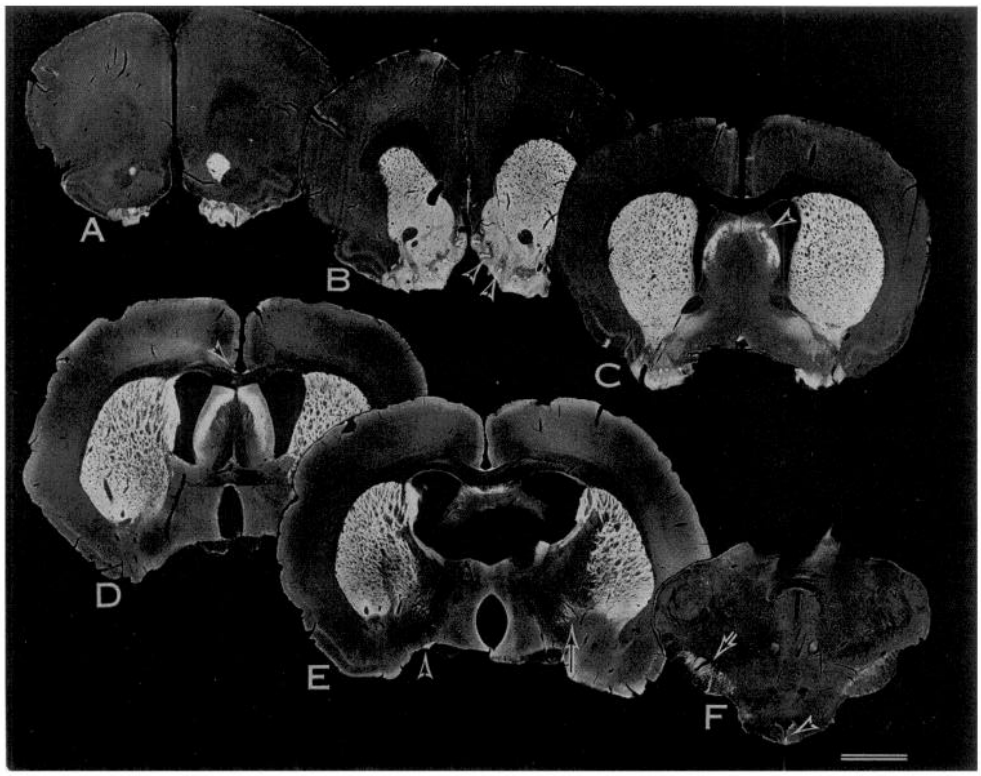


*Figure 3.* STEP immunoreactivity is strongest within basal ganglia and associated structures. Areas of intense immunoreactivity (*white areas*) correspond to nucleus accumbens and olfactory tubercle (*A*) and ventral striatum (*B*). Within the islands of Calleja, staining was also observed with a strongly stained central area in each island (*B, arrowheads*). Further caudally, caudatoputamen was stained as well as select neurons within the lateral septal nucleus (*C, arrowhead,* and *D*). Staining was also present within entorhinal and primary olfactory cortex (*A–C*). At more caudal levels of caudatoputamen, the striatonigral bundle was labeled (*E, arrow*) and could be followed to a terminal field within the substantia nigra pars reticulata (*F*). Additional staining was also present within the induseum griseum (*D, arrowhead*) and supraoptic nucleus (*E, arrowhead*). ABC-peroxidase staining, 50 μm-thick vibratome sections; photographic negative image. Scale bar, 2 mm.

may serve as substrate for serine/threonine phosphatases as well as PTPases, it was important to demonstrate that the phosphatase activity observed was specifically inhibited by tyrosine phosphatase inhibitors. Phosphatase activity was specifically inhibited by sodium vanadate ($IC_{50}$, 100 nM) and ammonium molybdate ($IC_{50}$, 2 μM), potent inhibitors of known tyrosine phosphatases. DARPP-32, a serine/threonine phosphatase inhibitor, had no effect on STEP phosphatase activity, as 5 μg (added to the 100 μl reaction) showed 99% phosphatase activity relative to STEP fusion protein activity. Neither poly(Glu-Tyr) (10 μM) nor heparin (100 μM) was inhibitory, as both resulted in, respectively, 108% and 89% of the STEP phosphatase activity. The enzymatic activity of STEP was compared to affinity-purified GST (Fig. 1), which showed no measurable phosphatase activity. STEP fusion protein was also able to dephosphorylate phosphotyrosyl α-casein in a time-dependent manner (P. J. Lombroso and B. Jena, unpublished observations).

In order to provide further evidence that the phosphatase activity observed was that of a PTP, a mutated STEP fusion protein was constructed and assayed for enzymatic activity. A single nucleotide was changed and resulted in a serine being present rather than a highly conserved cysteine, the latter being found only within tyrosine phosphatase domains and not within serine/threonine phosphatase domains. Phosphatase activity was abolished after this mutation (Fig. 1).

### *Characterization of STEP immunoreactive protein by Western blotting reveals a triplet of immunoreactive polypeptides*

A rabbit polyclonal antibody was affinity purified against GST-STEP fusion protein and used for all immunocytochemical experiments. As a first step in characterizing STEP immunoreactivity, Western blots of various tissues were performed. A major band at 46 kDa was seen in whole brain (lane 1, Fig. 2*A*), and was not detected in any of the tissues tested outside the CNS (lanes 2–5, Fig. 2*A*). No immunoreactive bands were observed in brain homogenates after preincubation of the antibody with STEP fusion protein (lane 6, Fig. 2*A*). In order to determine the regional distribution of immunoreactive polypeptides, protein homogenates from total brain, cerebellum, cerebral cortex, and striatum were compared (lanes 1–4, Fig. 2*B*). In striatal extracts, three polypeptide bands were detected with relative molecular weights ($M_r$) of 33 kDa, 37 kDa, and 46 kDa. The lower band (33 kDa) was detectable only within striatum, while the larger two bands were present in striatum and cerebral cortex. No detectable STEP immunoreactivity was observed in the cerebellum.

To investigate further the subcellular distribution of these immunoreactive polypeptides, phase separation of striatal homogenates was performed by Triton X-114 extraction. The results (Fig. 2*C*) indicate that STEP immunoreactivity remains in the aqueous phase (lane 1) and suggest that it is not strongly membrane associated (lane 2). In contrast, an integral membrane protein of synaptic vesicles, synaptophysin, is extracted by Triton X-114 pretreatment (lane 4, Fig. 2*C*). In addition, membrane preparations in the presence (150 mM NaCl) or absence of salt showed a similar enrichment of three bands only in cytosolic fractions, with the same molecular weights as those found by the phase separation experiments (data not shown).

### *STEP immunoreactivity is strongest within the basal ganglia and related structures*

Immunoperoxidase staining was performed on coronal and horizontal sections taken from adult rat brain in order to identify the major cell groups exhibiting STEP immunoreactivity. Photographic negative images of the stained sections are shown to illustrate the general patterns of immunoreactivity at low mag-

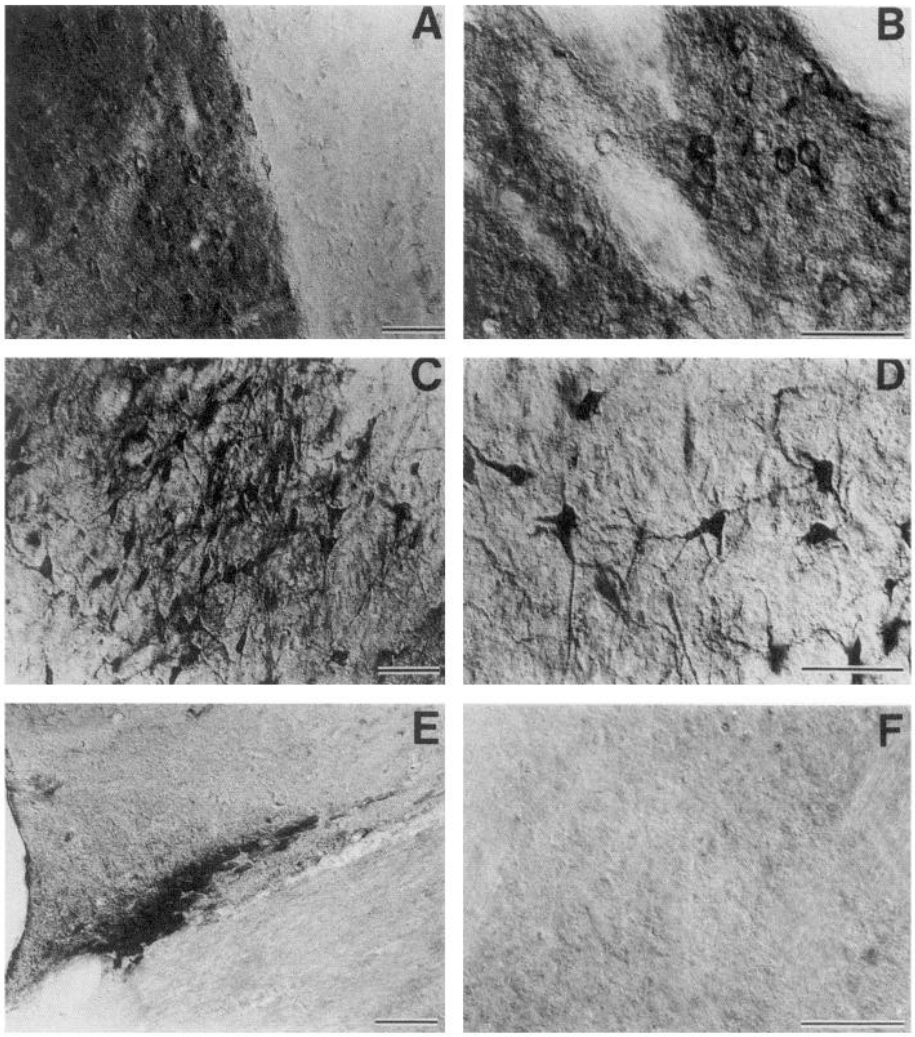


*Figure 4.* Photomicrographs showing STEP-immunoreactive neurons and neuropil in select brain regions. In striatum, STEP immunoreactivity was associated with both cell bodies and surrounding neuropil (*A, B*). In contrast, staining in the lateral septum was much more localized to neuronal bodies and dendrites (*C, D*). A mixture of cell body and neuropil staining was present in the induseum griseum (*E*). STEP immunoreactivity was abolished when sections were incubated in antiserum preblocked with STEP peptide (*F*). Scale bars, 75 μm.

nifications. The most rostral areas exhibiting strong STEP immunoreactivity included the nucleus accumbens and olfactory tubercle (Fig. 3*A*). At slightly more caudal levels, STEP immunostaining extended from the olfactory tubercle at the ventral surface of the brain into the overlying ventral caudatoputamen via bridges of immunoreactive neurons and their processes (Fig. 3*B*). Overall, weaker STEP immunoreactivity was seen throughout the islands of Calleja, except for small subregions which were intensely immunoreactive (Fig. 3*B*, arrowheads). At slightly more caudal levels, subsets of strongly immunoreactive neurons were found in the lateral septal nucleus (Fig. 3*C*, arrowhead), induseum griseum (Fig. 3*D*, arrowhead), and supraoptic nucleus (Fig. 3*E*, arrowhead). Weakly labeled axons and puncta were also present within the ventral pallidum (Fig. 3*C*) and in the substantia nigra, pars reticulata (Fig. 3*F*, arrow). In addition, weak STEP immunoreactivity was observed within neuronal cell bodies in the neocortex, cingulate cortex, piriform cortex, entorhinal cortex (Fig. 3*A–E*), the substantia nigra, pars compacta, and in tanycytes surrounding the third ventricle (Fig. 3*F*, arrowhead).

At higher magnifications, it was evident that both neurons and processes contributed to the intense staining of the caudatoputamen (Fig. 4*A,B*). Immunoperoxidase reaction product was present in the cytoplasm of neuronal cell bodies and proximal dendrites but was less prominent within nuclei (Fig. 4*B*). In addition, intense STEP immunoreactivity was present in fine processes comprising the neuropil, giving the appearance of a dense network of staining surrounding the immunoreactive cell bodies. The intensity of cellular and neuropil staining was strongest in dorsolateral portions of the caudatoputamen and diminished caudally. The boundary formed by the corpus callosum demarcated the dorsal and lateral extent of dense staining in striatum, with fewer neurons stained in areas of the neocortex.

In contrast to the patterns of STEP immunostaining in caudatoputamen, the cytoplasm of lateral septal neurons was intensely stained, with very little immunoreactivity in the surrounding neuropil (Fig. 4*C,D*). Intense cytoplasmic staining was also found within neurons in the induseum griseum (Fig. 4*E*) and supraoptic nucleus, without staining in the surrounding neuropil of these regions. Omission of primary antibodies or preincubation of STEP antiserum with purified STEP fusion protein, followed by the normal sequence of incubations in the ABC-peroxidase procedure and diaminobenzidine (DAB) and $H_2O_2$, abolished immunoreactive staining in these areas (Fig. 4*F*).

### *Immunocytochemical comparisons of STEP, DARPP-32, and TH*

The high density of STEP immunoreactive cells in brain regions known to receive dopaminergic inputs supported the hypothesis

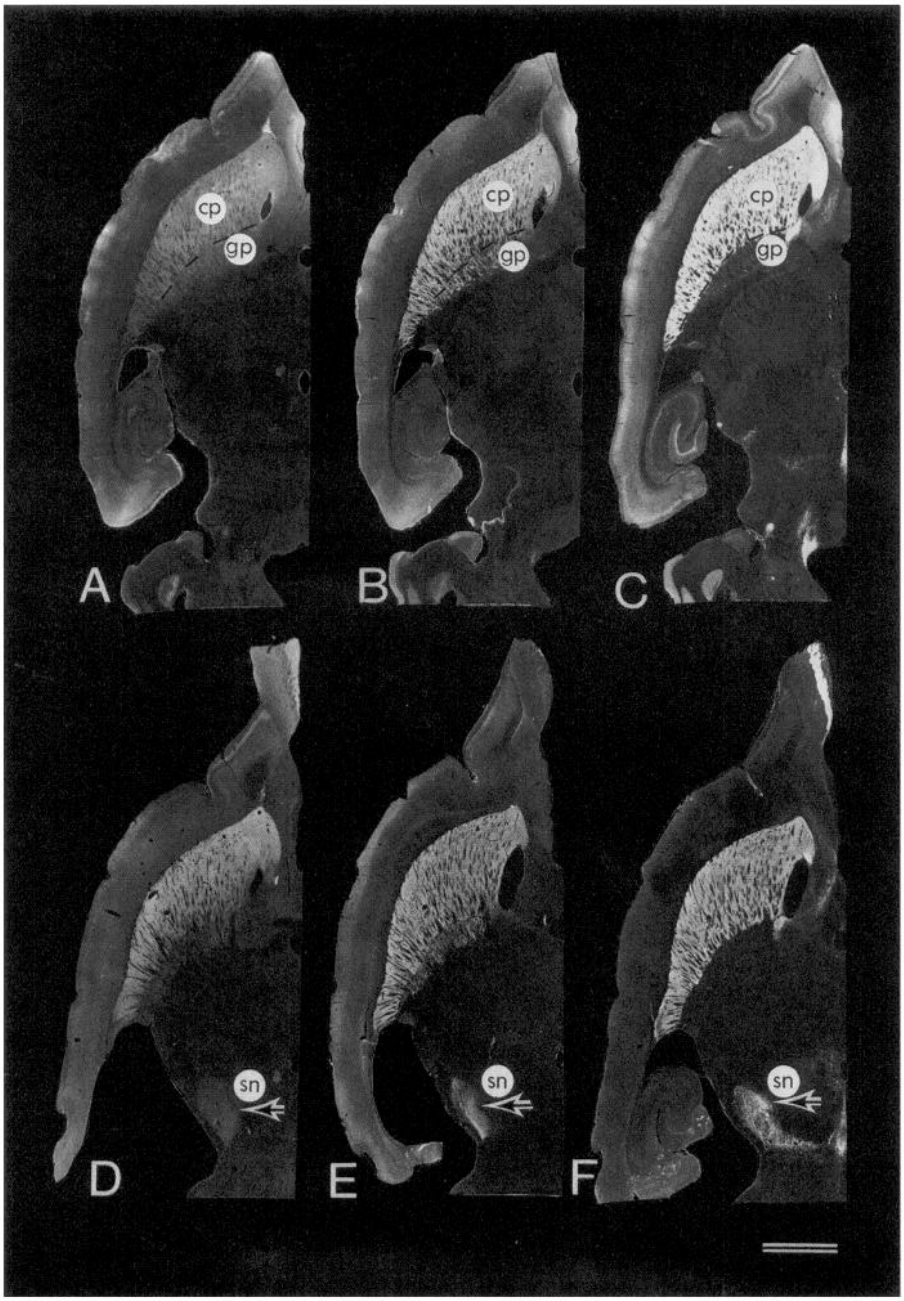


*Figure 5.* Low-power views of a series of horizontal sections comparing the distribution of STEP (*A* and *D*), DARPP-32 (*B* and *E*), and TH (*C* and *F*) immunoreactivies. STEP and DARPP-32 exhibit overlapping patterns while TH immunostaining shows partial overlap, particularly in the caudatoputamen, septum, and substantia nigra (*arrow*). *cp,* caudatoputamen; *gp,* globus pallidus; *sn,* substantia nigra. ABC-peroxidase staining, 40-μm-thick vibratome sections. Scale bar, 2 mm.

that STEP was expressed within dopaminoceptive neurons. In order to test this hypothesis, we compared the distribution of STEP immunoreactivity with two other well-characterized markers: the phosphoprotein DARPP-32, previously shown to be localized in dopaminoceptive neurons (Ouimet et al., 1984), and TH, a biosynthetic enzyme in the synthesis of dopamine. In Figure 5, the patterns of immunoreactive staining for STEP (Fig. 5*A,D*), DARPP-32 (Fig. 5*B,E*), and TH (Fig. 5*C,F*) are shown at two different levels in the brain sections cut in the horizontal plane, to allow comparisons of both caudatoputamen and substantia nigra staining within the same sections. The most striking result was the nearly complete overlap between STEP, DARPP-32, and TH immunoreactivity in the caudatoputamen (Fig. 5*A–F*). Since dopaminergic axons do not innervate the globus pallidus, this region lacks TH immunoreactivity (Fig. 5*C*). In contrast, immunostaining for both STEP and DARPP-32 was detected in axonal processes of the globus pallidus; these processes are likely to arise from immunoreactive neurons in the caudatoputamen that form the striatopallidal projection system (Fig. 5*D,E*; Ouimet et al., 1984).

The distribution of TH-immunoreactive cell bodies and processes within the substantia nigra was more extensive than the staining detected with STEP or DARPP-32 antibodies, as shown in Figure 5*D–F*. This result is in agreement with the previous findings of Ouimet et al. (1984), who showed that DARPP-32 is present in dopaminoceptive but not dopaminergic neurons. Although DARPP-32 immunoreactivity was generally stronger than STEP immunoreactivity in most regions, STEP showed a broader distribution within the substantia nigra (Fig. 5*D,E*). Additional differences could be detected in the intensity of staining with the three antibodies, particularly in the rostral caudatoputamen and in the nucleus accumbens, which exhibited

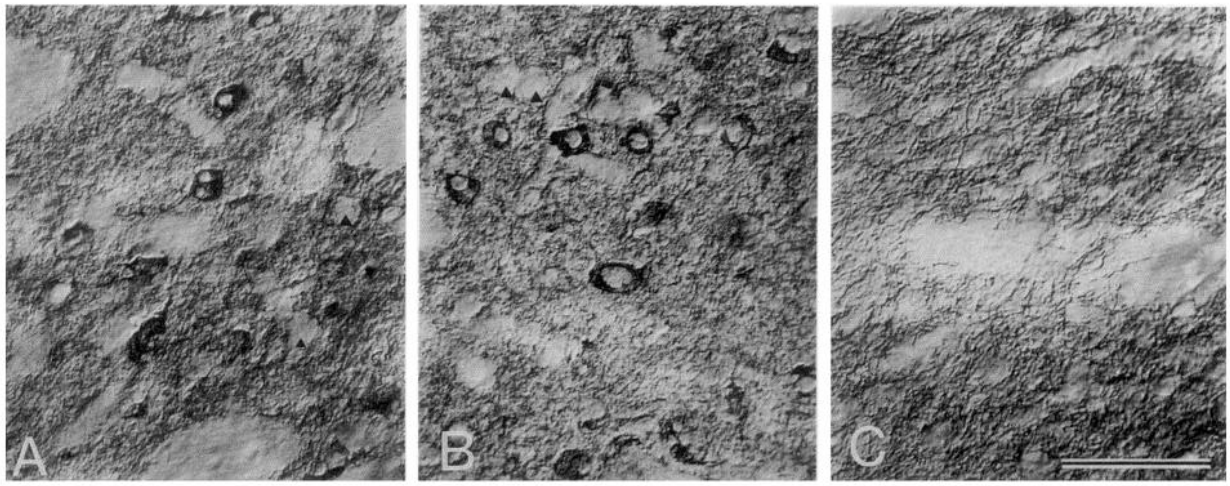


*Figure 6.* High-power views of immunoperoxidase labeling in striatum comparing STEP (*A*), DARPP-32 (*B*), and TH (*C*) immunoreactivity. STEP- and DARPP-32-immunoreactive neurons have similar distributions and morphology but are clearly present in a subset of neurons in the striatum, and several nonimmunoreactive neurons are shown (*arrowheads*). TH-immunoreactive axons terminate on striatal neurons, but there are no TH-immunoreactive cell bodies in the striatum (*C*). Scale bar, 75 μm.

very intense TH immunoreactivity (Fig. 5*C*). It is presently unclear whether these differences in the intensity of staining are due to differences in the affinity of the reagents or real differences in the intracellular concentrations of these three distinct proteins.

Examination of the staining in the caudatoputamen at higher magnifications indicated that the STEP- and DARPP-32-immunoreactive subsets contained neurons of approximately the same sizes and overall density (Fig. 6*A,B*). When viewed with Nomarski optics, subsets of caudatoputamen neurons were identified that were nonimmunoreactive with either STEP or DARPP-32 antibodies (Fig. 6*A,B,* arrowheads). Although the density of TH-immunoreactive axons and terminals was high in this region, there was a complete absence of any TH-immunoreactive cell bodies (Fig. 6*C*).

In order to determine whether STEP immunoreactive neuronal subsets received direct dopamine inputs, two-color immunofluorescent double-labeling studies were carried out with antibodies to STEP and TH. TH-immunoreactive synaptic boutons decorated the surfaces of STEP-positive neurons in the lateral septum (Fig. 7*A,* arrows), suggesting a synaptic relationship. In the striatum, the intensity and density of TH staining on cell bodies and within neuropil made distinct boutons more difficult to distinguish in this double-labeling paradigm. However, two-color immunofluorescent double-labeling studies demonstrated colocalization of STEP and DARPP-32 in striatal neurons (Fig. 7*B,C*). Counts of STEP-immunoreactive striatal neurons were made in two adjacent sections of the rat caudatoputamen. All DARPP-32–immunoreactive neurons were also STEP immunoreactive (50 of 50). Similarly, all STEP-immunoreactive neurons were also DARPP-32 positive (40 of 40). In the same sections, colocalization was not observed in STEP-immunoreactive neurons of the lateral septum. Taken together, these findings indicate STEP-immunoreactive polypeptide(s) are present in striatal and several other distinct subsets of neurons receiving dopaminergic inputs.

## Discussion

Phosphorylation of proteins on tyrosine residues involves three components: protein tyrosine kinases (PTKs), substrate phosphoproteins, and PTPs. Previous work has demonstrated that the first two of these components may show regional variations in the level of their expression within the CNS. Our results demonstrate that the third component may also exhibit a high degree of regional expression. The discussion will first review previous work on localization of PTPs and of their substrates, followed by a discussion of the results presented here.

A number of studies have demonstrated that some PTKs and their substrates show distinct patterns of expression both during development and in the adult CNS. Lai and Lemke (1991) have identified a family of CNS-specific PTKs, some of whose members were found within discrete brain regions and absent from others. Girault et al. (1992b) examined the level of tyrosine phosphorylated proteins in various brain regions and demonstrated regional variation of specific phosphoproteins over the course of development. Protein tyrosine phosphorylation was enhanced in cortical cultures grown in the presence of growth factors with known tyrosine kinase activities (Girault et al., 1992b). The level of tyrosine kinase activity in postsynaptic densities has also been shown to increase in a developmentally related manner (Gurd and Bissoon, 1990), and the level of tyrosine phosphorylation of substrate proteins was found to be highly variable, with an increase seen in some regions and a decrease seen in others.

Specific neurotransmitter pathways have also been examined for their relationship to protein tyrosine phosphorylation. Several proteins within the striatum are specifically phosphorylated on tyrosine residues after lesions of their dopaminergic input from the substantia nigra (Girault et al., 1992a). These results suggested that, within the striatum, tyrosine phosphorylation may play a role in mediating some of the postsynaptic effects of dopamine. The demonstration that PTP activity can be rapidly and specifically regulated by a G-protein coupled to the somatostatin receptor (Pan et al., 1992) provides one mechanism of regulating the level of phosphorylation of substrates. These experiments were conducted in neoplastic cells, and it is unclear whether similar mechanisms exist within the CNS. As a first step, it is important to determine whether PTPs exist within discrete nuclei of the brain and are postsynaptic to particular neurotransmitter receptors.

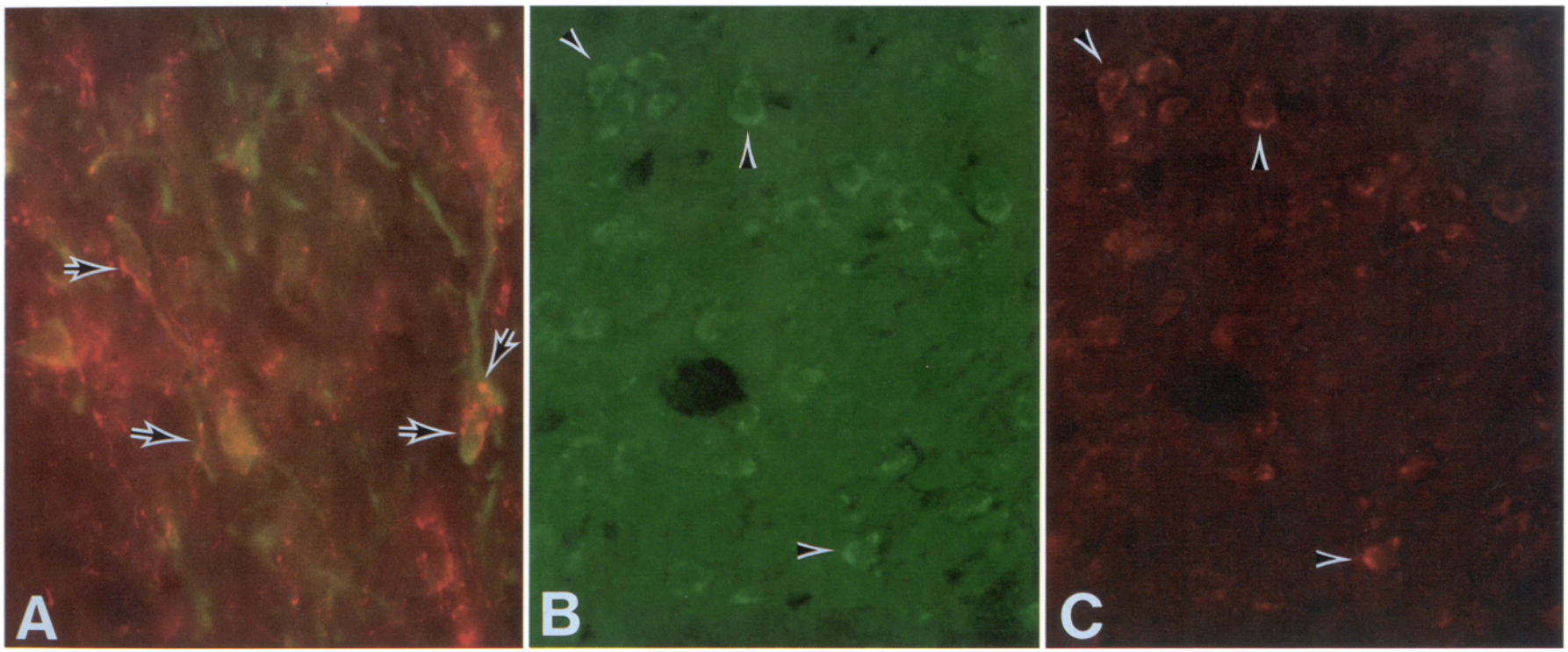


*Figure 7.* Two-color immunofluorescence staining in septal nucleus and caudatoputamen. *A*, Synaptic boutons immunoreactive for TH (rhodamine, *arrows*) were observed in close proximity with the cell bodies and dendrites of STEP-immunoreactive neurons (fluorescein) in the lateral septum. STEP-immunoreactive neurons (*B*; fluorescein) are also immunoreactive for DARPP-32 (*C*; rhodamine) in the striatum.

*STEP is distinct from other intracellular PTPs*

A number of features distinguish STEP from previously characterized intracellular PTPs. First, Western blots demonstrate that STEP immunoreactivity is found only within the CNS (Fig. 2*A*). Three bands are recognized by STEP antisera and these are enriched within the striatum. Only two of these bands are detectable within the cerebral cortex and none are detectable in the cerebellum (Fig. 2*B*). Tissue distribution of STEP immunoreactivity corresponds with the distribution of STEP mRNA previously demonstrated by Northern analysis (Lombroso, 1991). A second distinguishing feature is that STEP immunoreactivity is enriched in the soluble fractions, and does not appear to be associated with membrane fractions. Previous reports on other intracellular PTPs have either suggested or demonstrated an association with the membrane (Cool et al., 1990; Guan et al., 1990; Frangioni et al., 1992). This was demonstrated by both phase separation experiments and membrane preparations in the presence or absence of salt. Biochemical characterization also distinguishes STEP from other intracellular PTPs. STEP activity was specifically inhibited by sodium vanadate and molybdate, potent inhibitors of PTPs; however, unlike the intracellular PTPs, PTP1B, PTP5, and the nicotinic ACh receptor PTP (Tonks et al., 1988b; Jones et al., 1989; Mei and Huganir, 1991), STEP activity was not affected by heparin and poly(Glu-Tyr). Further work is in progress to characterize more fully the biochemical properties of STEP.

*In vitro* translation of STEP cDNA gives a translation product of 46 kDa, identical in size to the largest and most enriched of the three bands within the striatum (Lombroso, 1991). Several lines of evidence suggest that the three bands recognized by STEP antisera represent three separate proteins and are not degradation products of the same protein. First, identical bands are seen irrespective of the amounts of protease inhibitors added. To test this further, protease inhibitors were completely omitted in one series of experiments, and the same three bands were present in the various brain regions tested and with the same relative intensity. In addition, a panel of mAbs that have been generated against STEP are partially characterized. All of the mAbs that recognize bands on Western blots recognize a triplet with the same molecular weight as those presented in Figure 1 (P. J. Lombroso, J. R. Naegele, and G. Apicelli, unpublished observations). Further work will be needed to clarify whether these bands represent posttranslational modification(s) of the same STEP protein, products of alternative spliced STEP mRNAs (Lombroso, 1991), or three unrelated proteins that share a common epitope.

*A cysteine at position 300 is critical for phosphatase activity*

The catalytic domain of all PTPs isolated to date contains a canonical amino acid sequence (Charbonneau et al., 1989; Krueger et al., 1990). This sequence includes a cysteine residue that is thought to be necessary for catalytic activity. Mutational analysis of two receptor-like PTPs (CD45 and LAR) (Strueli et al., 1990) demonstrated a critical role for this highly conserved amino acid, and site-directed inactivation by iodoacetate (Pot and Dixon, 1992) provides additional support for the hypothesis that this cysteine is the nucleophile required for the removal of phosphate groups from substrate proteins. The results presented here support this hypothesis by demonstrating in an intracellular PTP that mutation of the equivalent cysteine at amino acid position 300 leads to the complete loss of phosphatase activity. Additional mutational analysis is required to characterize the relative importance of neighboring amino acid residues within the phosphatase domain, or within other domains of the STEP polypeptide.

*STEP immunoreactivity is in dopaminoceptive neurons*

STEP immunoreactivity within the adult rat striatum does not demonstrate histochemically distinct compartmentalization between striasomes and matrix. Biochemically specialized compartments were first described for AChE (Graybiel and Ragsdale, 1978), and have been subsequently reported for a number of striatal neurotransmitters and enzymes. In addition, many of the inputs to the striatum have been demonstrated to maintain their compartmental organization (Gerfen, 1989). In contrast, DARPP-32 and calcineurin, similar to STEP, do not exhibit compartmentalization in the adult rat brain (reviewed in Goto and Hirano, 1989; Graybiel, 1990).

The results presented here indicate that STEP immunoreactivity is generally not found within dopaminergic neurons themselves. The substantia nigra pars compacta, a nucleus composed chiefly of dopaminergic neurons, contains only a sparse, weakly stained subset of STEP-immunoreactive cell bodies, whereas the caudatoputamen, which lacks intrinsic dopaminergic cells, contained many STEP-immunoreactive neurons. The globus pallidus and substantia nigra pars reticulata are two areas that receive major projections from the striatum, and STEP immunoreactivity is confined to axonal processes within these nuclei, with little intrinsic neuronal staining.

In order to determine whether STEP-immunoreactive neurons were dopaminoceptive, we demonstrated overlapping populations of STEP-positive, TH-positive, and DARPP-32–positive neurons in the caudatoputamen. In a series of adjacent sections of rat brain incubated in antiserum against STEP, antibodies against DARPP-32, or antibodies specific for TH, we found a nearly perfect overlap in the three staining patterns within the striatum, strongly suggesting that STEP is highly enriched within dopaminoceptive neurons. Additional double-labeling experiments demonstrated that 100% of immunoreactive neurons in the caudatoputamen contained both STEP and DARPP-32 immunoreactivity. These findings indicate that STEP-immunoreactive neurons in the striatum, similar to DARPP-32–immunoreactive neurons, receive dopaminergic inputs.

Additional studies strongly suggested the presence of TH-positive synapses on STEP-immunoreactive neurons within the striatum and lateral septum. TH input was also present in the ventral striatum and the islands of Calleja, where a partial overlap was observed with STEP-immunoreactive cells and neuropil.

*STEP- and DARPP-32–immunoreactive subsets are mostly, but not entirely, overlapping*

Although the vast majority of striatal neurons contain both STEP and DARPP-32 immunoreactivity, this is not always the case in other brain regions, where cells tend to express STEP or DARPP-32, but not both. Several notable examples were found: first, in the lateral septal nucleus, STEP-immunoreactive neurons were numerous and intensely labeled but DARPP-32–immunoreactive neurons were rare or absent; second, in the neocortex, DARPP-32–immunoreactive pyramidal neurons were numerous in layer 6 but they exhibited little or no STEP immunoreactivity, which was present in neurons in layers 2+3

and 5; and, third, in the islands of Calleja, STEP immunoreactivity was present in cells and neuropil, while DARPP-32 immunoreactivity was absent.

Previous studies indicate that DARPP-32 is highly concentrated in those brain regions containing dense dopaminergic terminations (Hemmings et al., 1984; Walaas and Greengard, 1984b). Greengard and his coworkers found that DARPP-32 was not present in all dopaminoceptive neurons, but rather a subset that expressed the $D_1$ class of dopamine receptor (dopamine receptors coupled to adenylate cyclase) (Ouimet et al., 1984; Walaas and Greengard, 1984a). The $D_1$ receptor subtype has not been reported on neurons in the islands of Calleja, which are strongly STEP immunoreactive, suggesting either that $D_1$ receptor expression is not always linked to STEP expression, or that more sensitive techniques are needed to demonstrate this receptor type in the islands of Calleja. STEP does not appear to be found within all neurons receiving a dopamine input, since we have identified neurons in the medial septum that receive TH-positive terminations that are negative for STEP immunoreactivity, and neurons in the lateral septum that are strongly STEP immunoreactive that do not appear to receive dopaminergic projections. The TH-positive neurons, however, are in close proximity to the subset of lateral septal neurons that are strongly STEP immunoreactive, and dopamine could still act upon these neurons since dopamine and other monoamines are known to diffuse a considerable distance from the site of release (Reader et al., 1979). Additional studies need to be carried out to determine whether STEP-immunoreactive neurons are capable of responding physiologically to dopamine, and whether loss of dopaminergic inputs results in alterations in the levels of STEP. It will also be important to investigate whether these distinctions reflect differences in the distribution of distinct subclasses of dopamine receptors ($D_1$–$D_5$) on different neuronal subsets expressing STEP.

The neuronal subsets in which DARPP-32 and STEP do not colocalize are relatively rare. However, there is no evidence at the present time that they act through similar signal transduction pathways. DARPP-32, a serine/threonine phosphatase inhibitor, has no inhibitory effect on STEP enzymatic activity. Although STEP does possess several potential serine and threonine phosphorylation sites, the physiological significance of these sites is not yet known.

Many cytokines and growth factor receptors have been identified that are either directly or indirectly linked to tyrosine kinases. NGF, epidermal growth factor, platelet-derived growth factor, interleukin-4, and interferon-$\alpha$ are examples of some of the factors whose receptors exhibit tyrosine kinase activity (reviewed in Hunter and Cooper, 1985; Ullrich and Schlessinger, 1990; Mire-Sluis and Thorpe, 1991). One proposed mechanism for signal transduction via these neurotrophin receptors involves the induction of other genes, but until recently substrates had not been identified that linked tyrosine kinase activity at the cell surface to gene activation. It has now been demonstrated that interferon stimulates the tyrosine phosphorylation of several cytoplasmic transcription factors that rapidly translocate to the cell nucleus and stimulate transcription of various genes (David and Larner, 1992; Schindler et al., 1992).

In summary, STEP may regulate the activity of proteins phosphorylated in response to growth factor receptor or neurotransmitter receptor activation. It will be important in future work to determine whether STEP-immunoreactive neurons express unique types of receptors and to determine the cellular substrates of STEP. These studies will clarify whether the physiological role of this novel PTP is functionally linked to dopamine transmission or to separate signaling pathways.

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